R E M A R K S

Applicant's statement under 37 CFR 1.133 regarding the July 27, 2007 telephone discussion with Examiner Pande is set forth in the July 30, 2007 TRANSMITTAL OF COPY OF AMENDMENT UNDER 37 CFR 1.111 FILED MAY 4, 2007.

On August 28, 2007, the undersigned had a telephone interview with Examiner Pande and her supervisor, Examiner Fredman, to discuss the 35 USC 112, second paragraph rejection set forth in item no. 6 on pages 4 to 5 of the August 14, 2007 Office Action. The 35 USC 112 rejection concerned the terminology in claims 1 and 5 of "consisting essentially of."

During the aforesaid telephone interview, the Examiners agreed to withdraw the 35 USC 112 rejection. However, the Examiners said that the "Claim Interpretation" in item no. 7 on page 5 of the August 14, 2007 Office Action was being maintained.

The Examiner is respectfully requested to return copies of the IDS Forms filed on October 3, 2007, with the Examiner's initials in the left column next to each cited publication to indicate that the cited publications were considered and made of record.

The above amendment to each of claims 3 and 18 with respect to "Isogene" and "Ultraspec II" is supported in the specification on page 5, lines 27 to 29. "Isogene" is discussed in the enclosed copy of J. Periodontal Research, (2006), 41, 554-559. "Ultraspec II" is discussed in the Alexander and Raicht reference on page 265, right column, line 2.

Claim 4 was amended hereinabove to include a feature of claim 3.

Claims 1 and 5 were rejected under 35 USC 112, second paragraph, for the reasons set forth in item no. 6 on pages 4 to 5 of the August 14, 2007 Office Action.

This rejection concerns the amendments to claims 1 and 5 in the AMENDMENT UNDER 37 CFR 1.111 filed May 4, 2007, wherein "comprising" was replaced with --consisting essentially of.--

As discussed hereinabove, Examiners Pande and Fredman stated during the aforesaid August 28, 2007 telephone interview that the 35 USC 112, second paragraph rejection would be withdrawn.

It is further noted that claims 1 and 5 were amended hereinabove to replace "consisting essentially of" with --comprising--.

With respect to item no. 7 entitled "Claim Interpretation" on page 5 of the August 14, 2007 Office Action, as discussed above, the present claims 1 and 5 recite "comprising."

With respect to Rule 116, entry of the above amendments is respectfully requested, since the amendments are responsive to issues raised in the final rejection.

Claims 1 to 5 were rejected under 35 USC 102 as being anticipated by Alexander and Raicht (1998), Digestive Diseases and Sciences, Vol. 43, No. 12, pp. 2652-2658, as evidenced by Ultraspec™-II RNA, Isolation System, Biotecx Bulletin, No. 28, 1993, for the reasons in item no. 9 on pages 5 to 8 of the August 14, 2007 Office Action.

Claims 6 and 20 were rejected under 35 USC 103 as being unpatentable over Alexander and Raicht (1998), Digestive Diseases and Sciences, Vol. 43, No. 12, pp. 2652-2658, as evidenced by Ultraspec™-II RNA, Isolation System, Biotecx Bulletin, No. 28, (1993), in view of Sano et al., (1995), Cancer Research, 55:3785-3789 for the reasons set forth in item no. 11 on pages 8 to 10 of the August 14, 2007 Office Action.

It was admitted in the Office Action that Alexander and Raicht do not teach that the tumor marker is COX-2.

Claims 15 and 16 were rejected under 35 USC 103 as being unpatentable over Alexander and Raicht, (1998), Digestive Diseases and Sciences, Vol. 43, No. 12, pp. 2652-2658 as evidenced by Ultraspec™-II RNA, Isolation System, Biotecx Bulletin, No. 28, (1993) in view of Godfrey et al. (USP 7,101,663) for the reasons indicated in item no. 12 on pages 10 to 12 of the August 14, 2007 Office Action.

With respect to applicant's claim 15, it was admitted in the Office Action that Alexander and Raicht do not teach applicant's step d), namely, amplifying the cDNA from step c) being carried out by a nested PCR.

Alexander and Raicht teach a method for preparing a sample to extract RNA used in a tumor marker detecting method for diagnosing colon cancer consisting of homogenizing a collected biological sample in the presence of EDTA.

In contrast to Alexander and Raicht, applicant's present claims 1 and 5 recite the RNase inhibitor is not EDTA. Furthermore, applicant's present claims 3 and 18 recite that the

RNase inhibitor is selected from the group consisting of guanidine thiocyanate, Isogene and Ultraspec II, which are not taught or suggested by Alexander and Raicht.

Applicant's present claims are thus substantially different from Alexander and Raicht.

The Office Action takes the position that EDTA is a well-known chelating agent routinely used as an RNase inhibitor. However, RNase can be divided into two groups according to their dependence on bivalent cations for activity wherein one group includes those that have no specific requirement for these ions and the other group includes those that have an absolute requirement for a specific bivalent cation (see the enclosed copy of Wade and Robinson, Biochem. J., (1966), 101, 467 to 479). Further, the ribosomes from *Escherichia coli B* contain enzymes from both groups. Therefore, even if EDTA is used as an RNase inhibitor, EDTA cannot inhibit the activity of RNase having no specific requirement for a bivalent cation, and then a considerable part of RNA contained in a collected biological sample is rapidly digested.

According to applicant's present claims, a collected biological sample is homogenized in the presence of an RNase other than EDTA, such as guanidine thiocyanate, Isogene or Ultraspec™ II.

As seen in Example 2 on page 10 to 11 of the present specification, while no PCR products were obtained from a sample obtained by using the method of Alexander and Raicht, desired products containing high molecular weight RNAs, such as 28s and 18s rNAs were obtained from the sample obtained by using the method according to applicant's present claims.

Thus, applicant's claims have unexpectedly advantageous properties when compared with Alexander and Raicht.

The Ultraspec™ II RNA Isolation System, Biotecx Bulletin, No. 28 (1993) is directed only to a kit for the isolation of total RNA.

Sano et al. concern only COX-2 as a tumor marker.

USP 7,101,663 is directed only to a technique for carrying out RT-PCR.

It is respectfully submitted that one of ordinary skill in the art would not consider to combine the references in the manner as set forth in the Office Action. Even assuming *arguendo* that the references are combinable, it is respectfully submitted that combining the references as set forth in the Office Action would not lead to applicant's present claims.

In summary, it is respectfully submitted that applicant's present claims are not anticipated and are not rendered obvious over the references, either singly or combined in the manner relied upon in the Office Action, in view of the distinctions discussed hereinabove.

Withdrawal of each of the 35 USC 102 and 35 USC 103 rejections is thus respectfully requested.

Reconsideration is requested. Allowance is solicited.

If the Examiner has any comments, questions, objections or recommendations, the Examiner is invited to telephone the undersigned at the telephone number given below for prompt action.

Respectfully submitted,

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Encs.: (1) a copy of BioChem. J., (1966), 101, 467-479

(2) a copy of J. Periodontal Res., (2006), 41, 554-559

Biochem. J. (1966) 101, 467

Magnesium Ion-Independent Ribonucleic Acid Depolymerases in Bacteria

By H. E. WADE and H. K. ROBINSON
Microbiological Research Establishment, Porton, nr. Salisbury, Wilts.

(*Received* 10 *March* 1966)

The distribution of ribonucleases among bacteria has been determined from the examination of a wide variety of species. Bacteria that had been growing rapidly on a solid medium were harvested, treated with acetone and incubated in the presence of EDTA between pH 4 and pH 9. The ribonuclease activity was determined from the rate at which acid-soluble nucleotides were released. Out of nearly 200 strains examined, about 30 did not contain a detectable ribonuclease. The pH optima of ribonucleases in the remainder were sufficiently distinctive to suggest a use in taxonomy. *Escherichia coli* B was examined in more detail to determine the factors responsible for variations in the ribonuclease content of this bacterium. Growth rate had little influence on ribonuclease content when a complex medium containing no readily assimilable carbohydrate was used; the addition of glucose resulted in a marked increase in ribonuclease and a dependence of enzyme content on growth rate. An increase in the concentration of sodium chloride in the medium decreased the ribonuclease content of bacteria growing on it.

Enzymes that depolymerize RNA can be divided into two groups according to their dependence on bivalent cations for activity. One group includes those that have no specific requirement for these ions and usually degrade RNA initially to nucleoside 2′,3′-(cyclic)-phosphates without an initial hydrolytic cleavage (ribonucleases); the other includes enzymes that have an absolute requirement for a specific bivalent cation, commonly Mg^{2+}, and degrade RNA directly by hydrolysis (phosphodiesterase), phosphorolysis (polynucleotide phosphorylase) or pyrophosphorolysis (polynucleotide pyrophosphorylase), usually into nucleoside 5′-phosphates or their derivatives.

The ribosomes from *Escherichia coli* B contain enzymes from both groups (Wade, 1961; Spahr & Hollingworth, 1961; Wade & Lovett, 1961; Spahr, 1964) but, when they are incubated in the presence of EDTA, the ribonuclease alone is active and the progress of its activity can be followed by measuring the release of acid-soluble nucleotides. Its activity can also be followed in unfractionated suspensions of disrupted bacteria (Wade, 1961). During an examination of other species, *Pseudomonas fluorescens* N.C.I.B. 8248 was found to be deficient in such an enzyme (Wade & Robinson, 1963).

The present paper describes the general distribution of this type of enzyme among bacteria. Although the original intention was to carry out a qualitative survey, scoring only the presence or absence of ribonuclease, exploratory experiments with *E. coli* B suggested that a rough quantitative measurement of ribonuclease activity could be obtained that would permit a more precise comparison between different species of bacteria.

The results showed that different species display differences in content and properties of ribonuclease that broadly follow the recognized divisions in bacterial classification (Breed, Murray & Smith, 1957).

MATERIALS AND METHODS

Organisms. Bacteria were obtained from the following collections: American Collection of Type Cultures, Rockville, Md., U.S.A. (A.T.C.C.); Boots Pure Drug Co. Ltd. Research Department, Antibiotic and Fermentation Division, Nottingham (F.D.); International Collection of Phytopathogenic Bacteria (I.C.P.B.); Microbiological Research Establishment, Salisbury (M.R.E.); National Collection of Dairy Organisms, National Institute for Research in Dairying, Reading (N.C.D.O.); National Collection of Industrial Bacteria, Torry Research Station, Aberdeen (N.C.I.B.); National Collection of Marine Bacteria, Torry Research Station, Aberdeen (N.C.M.B.); National Collection of Plant Pathogenic Bacteria, Harpenden, Herts. (N.C.P.P.B.); National Collection of Type Cultures, Central Public Health Laboratory, London (N.C.T.C.); Rothamsted Experimental Station, Harpenden (R.E.S.).

Pseudomonas fluorescens KB1 was obtained from Dr Margot Kogut (University of Sheffield, England) and

Table 1. *Composition of media*

Concentrations of medium constituents are in g./100g. of medium. When Oxoid media or medium constituents are listed their identification is given in parentheses. The type and concentration of agar have been excluded. Other minor constituents are present in sensitivity-test agar and reinforced clostridial agar.

	Plate-count agar (CM183)	Dextrose-peptone agar (CM13)	Tomato juice-agar (CM113)	Whey-agar (CM125)	Malt extract-agar (CM59)	Milk-agar (CM21)	Sensitivity-test agar (CM215)	Nutrient agar (CM3)	Blood base-agar (CM271)	Reinforced clostridial agar (CM151)	Tryptone-soya-agar (CM131)	Lister peptone-agar	Lab-Lemco peptone-yeast extract-agar
Bacteriological peptone (L37)	—	2	1	1	—	0·5	—	0·5	—	1	—	—	1
Mycological peptone (L40)	—	—	—	—	0·5	—	—	—	—	—	—	—	—
Peptonized milk (L32)	—	—	1	—	—	—	—	—	—	—	—	—	—
Tryptone (L42)	0·5	—	—	—	—	—	—	—	—	—	1·5	—	—
Soya peptone (L44)	—	—	—	—	—	—	—	—	—	—	0·5	—	—
Protease peptone (L46)	—	—	—	—	—	—	1	—	1·5	—	—	—	—
Peptone (Evans)	—	—	—	—	—	—	—	—	—	—	—	1	—
Milk solids	—	—	—	—	—	0·1	—	—	—	—	—	—	—
Dehydrated whey	—	—	—	1·3	—	—	—	—	—	—	—	—	—
Tomato-juice solids	—	—	2	—	—	—	—	—	—	—	—	—	—
Malt extract (L39)	—	—	—	—	3	—	—	—	—	—	—	—	—
Yeast extract (L20 or L21)	0·25	—	—	—	—	0·3	—	0·2	0·5	0·3	—	—	0·4
Lab-Lemco beef extract (L30)	—	—	—	—	—	—	—	0·1	—	1	—	—	1
Veal-infusion solids	—	—	—	—	—	—	1	—	—	—	—	—	—
Liver digest (L27)	—	—	—	—	—	—	—	—	0·25	—	—	—	—
Glucose	0·1	1	—	—	—	—	1	—	—	0·5	—	—	—
Mannitol	—	—	—	—	—	—	—	—	—	—	—	1	—
NaCl	—	0·5	—	—	—	—	0·3	0·5	0·5	0·5	0·5	0·5	0·5

Pseudomonas putida C–1B from Mr D. W. Tempest (Microbiological Research Establishment, Salisbury). *Proteus mirabilis* PR–27 was obtained from Professor Judith F. M. Hoeniger (University of Toronto, Canada). *Micrococcus lysodeikticus* Delft 538 was obtained from Dr A. C. Baird-Parker. *Escherichia coli* w 3110 was obtained from Dr Esther M. Lederberg (University of Stanford, U.S.A.). *Escherichia coli* C6 and several other strains of the C series and IR series were obtained from Dr N. P. Burman (Metropolitan Water Board, London). The ribonuclease-deficient strain of *E. coli* (Table 4) was obtained originally from the National Collection of Type Cultures (N.C.T.C. 8164). In the light of evidence at that time that this property was extremely uncommon in this species, it seemed likely that the strain had mutated. To avoid confusion, the strain was identified as M.R.E. 600 (Cammack & Wade, 1965; Wade & Robinson, 1965*a*,*b*). It is now known that the original isolate C6 was in fact deficient in ribonuclease and the preparation available from The National Collection of Industrial Bacteria (N.C.I.B. 9270) deficient also.

Culture conditions. Bacteria were normally grown on a nutrient agar in Petri dishes (9 cm. diam.). The influence of the medium on the synthesis of ribonuclease by *Escherichia coli* B was examined by growing these bacteria on the media listed in Table 1, on a yeast extract–glucose–agar that contained 2·5% (w/v) of Yeatex (light-grade yeast extract from The Trent Yeast Extract Co. Ltd.) and on a glucose–ammonia–salts–agar. *Acetobacter oxydans* was grown on a yeast extract–malt extract–agar that contained 0·5% of yeast extract (Difco), 0·5% of malt extract (Difco), salts and glucose at pH 6. *Azotobacter chroococcum* was grown on a defined medium (Norris & Jensen, 1957).

For the routine determination of ribonuclease activity, bacteria were spread on the surface of a nutrient agar, usually plate-count agar (Oxoid), incubated and harvested by scraping with the flat end of a spatula. With fast-growing species a temperature 5–15° lower than the optimum was used for a period of more than 18 hr. or the period of incubation was decreased to 4–8 hr. to avoid excessive growth. It was important to obtain bacteria in a growing state to ensure an adequate concentration of endogenous RNA on which the enzyme could act. In those instances where encroachment of the bacteria into the nutrient agar made recovery difficult, cellophan was spread on the surface before seeding.

The comparison between acetone-extracted bacteria and disrupted bacteria (Fig. 6) was carried out with bacteria that had been grown on liquid media by continuous culture (Wade & Robinson, 1965*a*).

Preparations of *Pasteurella* that had been grown on a liquid medium (Burrows & Bacon, 1954) were obtained from Dr T. W. Burrows. A preparation of *Brucella suis* was obtained from Dr J. Keppie and a preparation of *Listeria monocytogenes* from Mr S. Peacock.

Preparations of *Aerobacter aerogenes* N.C.T.C. 418 that had been grown on a glycerol–ammonia–salts medium by continuous culture under conditions of carbon, nitrogen or sulphur limitation (Tempest, 1965) were obtained from Dr D. Herbert. Preparations that had been growing under conditions of phosphorus, magnesium or potassium limitation were obtained from Mr D. W. Tempest.

Ribonuclease activity. The bacteria were spread over the surface of nutrient agar in three to 12 Petri dishes (about 2×10^8 bacteria in each dish), after at least one passage through the medium, and grown under conditions that would ensure a satisfactory yield of growing bacteria. The harvested bacteria were suspended in 5 ml. of a solution containing NaCl (0·145 M) and $MgCl_2$ (5 mM) in a tapered graduated 10 ml. centrifuge tube at 0–2° and treated with 5 ml. of acetone. The tube was stoppered and stored for up to 1 month at −20° until required.

The acetone-extracted cells were sedimented in a swing-out bench centrifuge (MSE Minor) at 4000 rev./min. for 15 min. The volume of the packed bacteria was noted and the supernatant discarded. Suction from a water pump removed the remaining acetone from the deposit. The bacteria were resuspended in a solution containing NaCl (0·145 M) and $MgCl_2$ (5 mM) (10–20%, wet vol.) at 0–2°. With most species, three Petri dishes provided about 0·5 ml. (wet vol.) of packed acetone-extracted bacteria. The suspension (0–2°) was mixed thoroughly and adjusted to about pH 8·5 with Na_2CO_3, with phenolphthalein paper as an external indicator.

A 0·2 ml. volume of this suspension was mixed with 0·47 ml. of 0·4 N-KOH and left at 37° for 1·5–2 hr. to hydrolyse the RNA to acid-soluble products and provide a measure of the total RNA present. Longer periods of exposure resulted in a proportion of the DNA in some species appearing in the acid-soluble fraction, thus inflating the estimate of RNA. Two samples of the product (0·05 ml.) were delivered separately on to filter paper 1 cm. wide, drawn from a roll of Whatman no. 542 chromatography paper. The length of paper (about 8 cm.) that each occupied was cut off and immersed in 10 ml. of 0·1 N-$HClO_4$ at room temperature (Wade, 1961).

Two 0·05 ml. volumes of the suspension of acetone-extracted bacteria were delivered directly on to filter paper and similarly treated with acid to provide a measure of naturally occurring acid-soluble nucleotides and bases already present.

A 0·15 ml. volume of the suspension of acetone-treated bacteria was delivered at recorded times into each of six test tubes (4 in. × 0·5 in. diam.) at 37° that contained 0·35 ml. of 0·13 M-veronal–acetate buffer (Roth, 1954) containing EDTA (28·5 mM) (veronal–acetate–EDTA buffer) at pH values 4, 5, 6, 7, 8 and 9 respectively. Samples (0·05 ml.), taken after incubation for 5, 10, 30 and 60 min., were delivered on to filter paper and immersed in acid as described above.

Two untreated strips of filter paper were extracted directly with acid to estimate the ultraviolet-absorbing substances in the paper (paper blank).

The tubes that contained filter-paper strips in acid were inverted after intervals of 0·5 hr. and 1 hr. After 2–4 hr. the acid from each was poured into a 4 cm. cell of the Unicam SP. 500 spectrophotometer (Cambridge Instruments Ltd., Cambridge) and the extinction at 260 mμ measured against the acid used. In a few instances the bacteria failed to adhere adequately to the filter paper and a slightly turbid extract was obtained. Under these circumstances, the acid extract was cleared by centrifuging or, if the interference was slight, a rough correction was made by measuring the extinction at 320 mμ and subtracting this value from the one obtained at 260 mμ.

The increase in extinction ($E^{4cm}_{260m\mu}$) due to the activity of the ribonuclease was obtained by subtracting the extinction of the direct acid extract (adjusted to allow for its lower dilution factor) from that of the acid extract after

incubation. The resulting values were then corrected for the paper blank, which did not usually exceed 0·03 ($E^{4cm.}_{260m\mu}$), and the extinction due to the buffer, which was also about 0·03 ($E^{4cm.}_{260m\mu}$).

A graph of the quantity of nucleotides released plotted against pH established the optimum for the enzyme. The maximum rate of nucleotide released at this pH value provided a rough measure of the ribonuclease activity. Extinction measurements were finally converted into nucleotide concentration by using a mean molar extinction (ϵ) 10^4 and the results from different species were normalized by basing them on the volume of acetone-extracted bacteria.

The determination of deoxyribose by a sensitive diphenylamine method (Burton, 1956) carried out on the acid extracts of several representative strains of bacteria showed that the nucleotides produced during incubation do not originate from the DNA.

Dry weight. A volume (0·5 ml.) of suspension containing 10–20 mg. dry wt. of material was treated with 4·5 ml. of acetone and centrifuged in a weighed test tube (4 in. × 0·5 in. diam.). The supernatant was discarded and the drained deposit was dried at 105° for 18 hr. Determinations were carried out in duplicate.

RESULTS

The choice of growth conditions was largely governed by a requirement for bacteria that contained sufficient RNA to serve as a substrate for the ribonuclease. Agar-based media were used in preference to liquid media since these provided the most convenient method of growing and recovering the quantity of bacteria required. Most of the species demanded a complex medium for rapid growth. The possibility that ribonuclease in these media (Table 1) would interfere with the assessment of bacterial ribonuclease was examined with the ribonuclease-deficient *Pseudomonas fluorescens* N.C.I.B. 8248.

Bacteria that had been grown on the different media were extracted with 50% (v/v) acetone and incubated at 37° in veronal–acetate–EDTA buffers at pH 5, 7 and 9. No significant release of nucleotide could be detected after incubation for 4 hr. The low concentrations of ribonuclease in the constituents of the media were demonstrated by incubating dialysed preparations (dialysed to diminish the concentrations of acid-soluble ultraviolet-absorbing substances already present) with a ribosome preparation (6 mg. dry wt./ml.) from *Ps. fluorescens* in veronal–acetate–EDTA buffer, pH 4–9. Casein acid hydrolysate, Lab-Lemco beef extract, liver digest, mycological peptone, proteose peptone, tryptone, soya peptone and yeast extract (Table 1) degraded RNA at a rate of less than 5 μmoles of nucleotide/g. dry wt. of undialysed material/hr. Bacteriological peptone and malt extract had activities of 12 and 19 μmoles of nucleotide/g./hr. respectively. Pancreatic ribonuclease was retained by the dialysis sac when similarly dialysed.

It is probable that the various treatments to which the constituents are subjected during their preparation and the final autoclaving of the complete medium are sufficient to decrease the activity of ribonuclease to insignificant levels.

In addition to interference from contaminating ribonuclease, it was also necessary to consider the possible influence of the various media on the synthesis of the bacterial enzyme. This was examined by growing a ribonuclease-containing strain, *E. coli* B, on several different media and measuring the ribonuclease activities of the harvested bacteria.

Each medium was seeded with bacteria (about 2×10^8 bacteria in each Petri dish) and incubated at 37° for 7 hr. The ribonuclease activities of the different preparations of bacteria were then compared by following the release of acid-soluble nucleotides during the incubation (37°) of acetone-extracted bacteria in veronal–acetate–EDTA buffer, pH 7.

The results (Fig. 1) suggested that the composition of the medium does in fact influence the ribonuclease content of the bacteria. It was also observed, however, that in those preparations which showed the lowest activity the mean cell size of the harvested bacteria was lower and the yield of bacteria higher, suggesting that the differences in enzyme activity reflected differences in the physiology of the bacteria at the time of harvesting. On the richer media, e.g. blood base–agar and Lab-Lemco peptone–yeast extract–agar, the cultures may have approached more closely to maturity and a declining growth rate after this fixed period of growth than cultures growing on the poorer media, e.g. plate-count agar and dextrose–peptone–agar.

The relationship between the period of growth and the ribonuclease activity was therefore studied in more detail with plate-count agar by using the concentration of RNA as an indication of the growth rate. When bacteria harvested from plate-count agar after different periods of growth were extracted with 50% acetone and incubated in veronal–acetate–EDTA buffer, pH 7, nucleotides were released at a uniform rate after a lag of about 5 min., up to a point at which about 60% of the total RNA had been degraded (Fig. 2 and Table 2). As the period of growth was increased from 3 hr. to 16 hr. (when the culture was fully grown), the ribonuclease activity diminished (Fig. 2). This decrease was not sufficiently great, however, to account entirely for the low activities observed in bacteria grown on some of the richer media (Fig. 1).

Since a decrease in the concentration of Mg^{2+} relative to univalent cations encourages the loss of ribonuclease from spheroplasts of *E. coli* (Neu & Heppel, 1965) and ribosomes (Spirin, Kisselev, Shakulov & Bogdanov, 1963), another possible explanation was that sodium chloride, included in

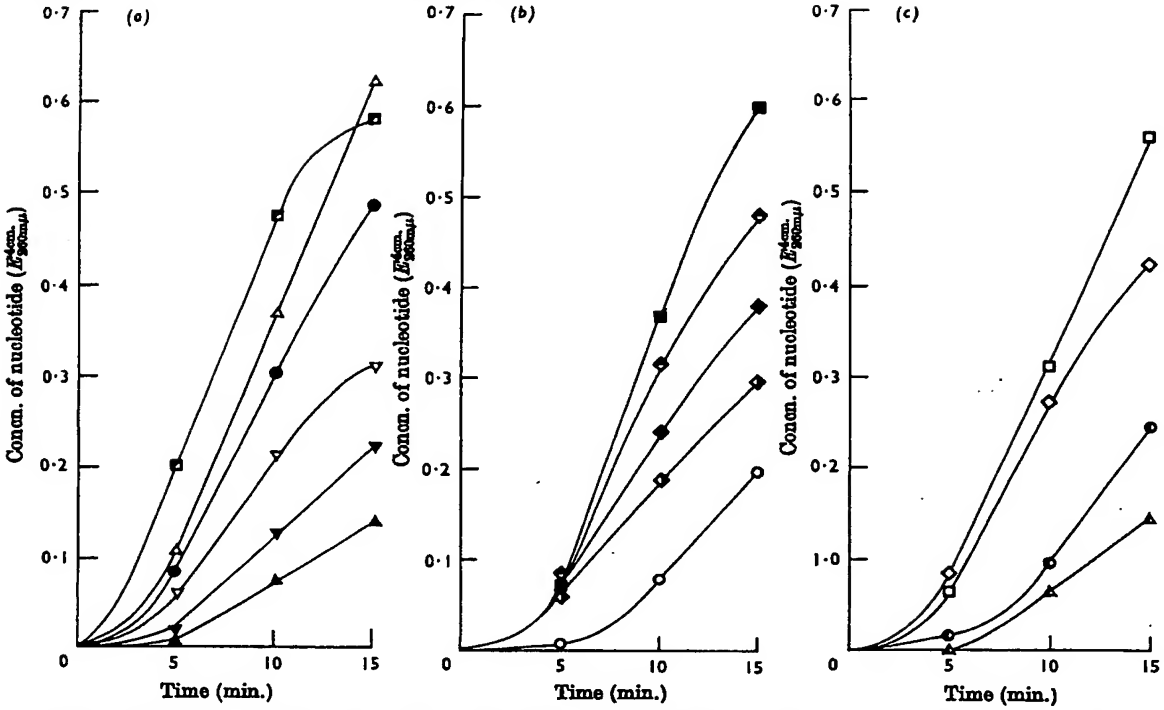

Fig. 1. Ribonuclease activity of *E. coli* B grown for 7 hr. at 37° on several nutrient agars. (*a*) Lister peptone–agar (◩), malt extract–agar (△), plate-count agar (●), milk–agar (▽), a glucose–ammonia–salts–agar (▼), blood base–agar (▲); (*b*) tomato juice–agar (■), dextrose–peptone agar (◈), sensitivity-test agar (◆), nutrient agar (◈), Lab-Lemco peptone–yeast extract–agar (○); (*c*) reinforced clostridial agar (□), whey–agar (◇), a yeast extract–agar (◐), tryptone–soya–agar (▲). The harvested bacteria were extracted with 50% acetone and incubated (0·06 ml. wet vol. of bacteria/ml.) at 37° in veronal–acetate–EDTA buffer, pH 7. Samples (0·05 ml.) were extracted with 10 ml. of 0·1 N-$HClO_4$. The extinction (260 mμ) of the extract provided a measure of acid-soluble nucleotide released.

some media (Table 1), lowered the relative concentration of Mg^{2+} and encouraged the loss of ribonuclease from the bacteria. The effect of adding sodium chloride to plate-count agar was therefore examined.

The addition of sodium chloride (0·2 M) had no effect on the growth rate or the concentration of RNA but resulted in a decrease in ribonuclease activity and, after a period of 16 hr. of growth, a more pronounced lag in the release of nucleotides (Fig. 2 and Table 2). In both these respects bacteria grown on the supplemented plate-count agar more closely resembled those grown on blood base–agar and Lab-Lemco peptone–yeast extract–agar. The results of several individual experiments confirmed the effects of supplementing plate-count agar with sodium chloride (Fig. 3).

Although a higher salt concentration in the medium decreased the activity of bacterial ribonuclease, secreted ribonuclease could not be detected in the 50%-acetone extract of bacteria that had been grown on the supplemented medium even when diffusion from the bacteria during growth was restricted by cellophan. Further, an attempt to suppress the suspected loss of ribonuclease from bacteria growing on blood base–agar or Lab-Lemco peptone–yeast extract–agar by supplementing the medium with magnesium chloride (10 mM) was unsuccessful.

The results at this time had suggested that the concentration of ribonuclease in *E. coli* B diminishes with the growth rate and is generally lowered by increasing the concentration of sodium chloride in the medium. However, neither of these factors could account for some of the differences observed. They did not explain, for example, why bacteria from reinforced clostridial agar have a high ribonuclease activity whereas bacteria harvested after the same period of incubation from the very similar Lab-Lemco peptone–yeast extract–agar (Table 1)

have not (Fig. 1). Cysteine, which is present in the former (0·5%), was not responsible, since its addition to plate-count agar, Lab-Lemco peptone–yeast extract–agar or blood base–agar had no effect on the ribonuclease activity in *E. coli* B.

An explanation for the major differences observed in Fig. 1 came from a closer examination of *E. coli* B growing on six media, three of which (plate-count agar, Lister peptone–agar and reinforced clostridial agar) produced bacteria with high ribonuclease activity and three of which (blood base–agar, Lab-Lemco peptone–yeast extract–agar and nutrient agar) produced bacteria with low ribonuclease activity (Fig. 1).

The bacteria were grown at 37° for different times (3–22 hr.) on each medium. The harvested bacteria were extracted with 50% acetone and incubated at 37° in veronal–acetate–EDTA buffer, pH 7.

As the yield of bacteria increased, the concentra-

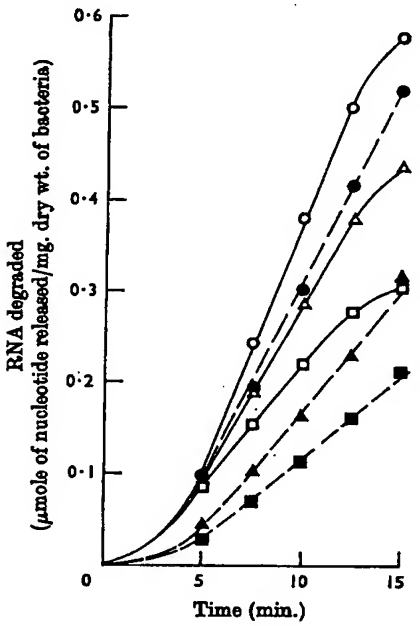

Fig. 2. Effect of higher NaCl concentration on the ribonuclease activity of *E. coli* B growing on plate-count agar. Bacteria were grown in the absence (○, △, □) or presence (●, ▲, ■) of 0·2 M-NaCl at 37° for 3 hr. (○, ●), 5 hr. (△, ▲) or 16 hr. (□, ■), harvested, extracted with 50% acetone and incubated at 37° with veronal–acetate–EDTA buffer, pH 7. The concentrations of RNA in these preparations are given in Table 2.

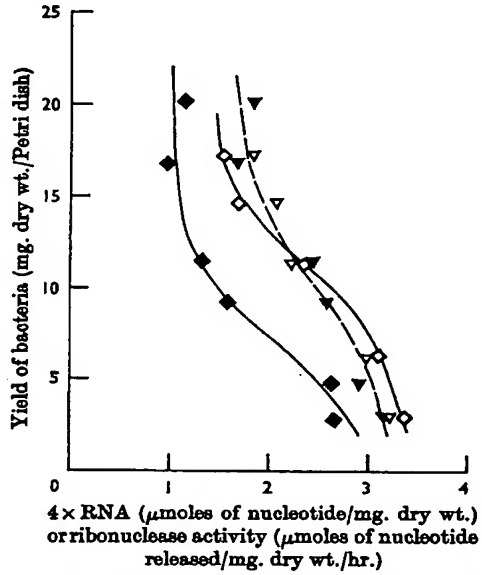

Fig. 3. Effect of higher NaCl concentration on the relationship between the concentrations of RNA (▽, ◇) and ribonuclease (▼, ◆) in *E. coli* B growing at 37° for 3–16 hr. on plate-count agar in the absence (▽, ▼) or presence (◇, ◆) of 0·2 M-NaCl. The harvested bacteria were extracted with 50% acetone and incubated at 37° in veronal–acetate–EDTA buffer, pH 7.

Table 2. *Yield and RNA content of* E. coli *B grown on plate-count agar*

Bacteria were grown in the absence or presence of 0·2 M-NaCl at 37° on Petri dishes. The ribonuclease activities of the same preparations are illustrated in Fig. 2.

Medium	Period of growth (hr.)	Yield of bacteria (mg. of dry wt./Petri dish)	RNA content (μmole of nucleotide/mg. dry wt. of bacteria)
Plate-count agar	3	3	0·8
	5	11	0·5
	16	17	0·46
Plate-count agar with NaCl (0·2 M)	3	2·8	0·8
	5	11·5	0·61
	16	20	0·46

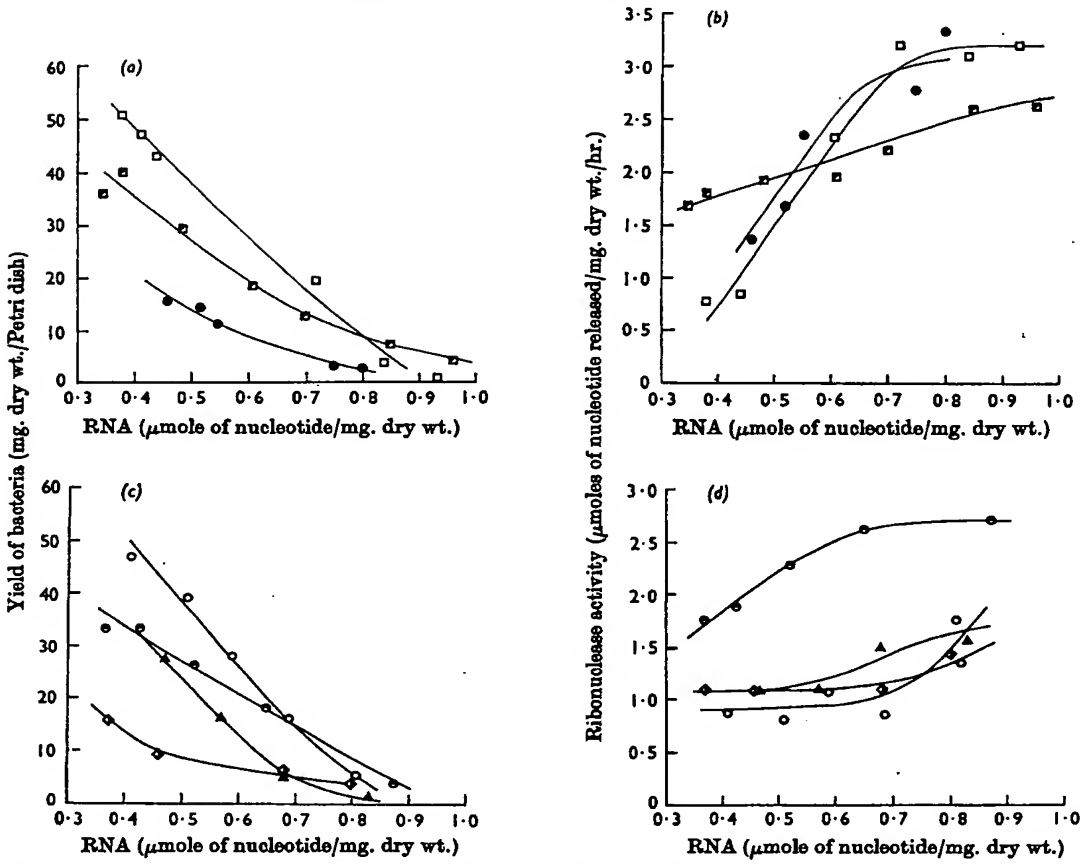

Fig. 4. Effects of growth conditions on the ribonuclease content of *E. coli* B. The bacteria were grown at 37° for 3–22 hr. on (*a* and *b*) reinforced clostridial agar (□), Lister peptone–agar (◩) and plate-count agar (●) or on (*c* and *d*) blood base–agar (▲), nutrient agar (◆), and Lab-Lemco peptone–yeast extract–agar in the absence (○) or presence (◓) of 0·5% glucose. The bacteria were extracted with acetone and incubated in veronal–acetate–EDTA buffer, pH 7, at 37°. The ribonuclease activity was determined from the rate at which acid-soluble nucleotides were released.

tion of RNA diminished (Figs. 4*a* and 4*c*). The changes in ribonuclease activity showed the expected trend: as the RNA concentration diminished (with increasing maturity of the culture and decreasing growth rate) the ribonuclease activity diminished (Figs. 4*b* and 4*d*). There were, however, marked quantitative differences between the different preparations of bacteria. The ribonuclease activity of bacteria growing on plate-count agar, Lister peptone–agar or reinforced clostridial agar was high at fast growth rates and underwent a marked decrease as the growth rate diminished (Fig. 4*b*), whereas the ribonuclease activity of bacteria growing on blood base–agar, Lab-Lemco peptone–yeast extract–agar or nutrient agar was low and hardly affected by the growth rate (Fig. 4*d*).

One feature that was common to the media in the first group (Figs. 4*a* and 4*b*) was the possession of a readily assimilable source of energy provided by low-molecular-weight carbohydrate. With the two media that contained glucose (plate-count agar and reinforced clostridial agar) and to a smaller extent the medium that contained mannitol (Lister peptone–agar) much higher ribonuclease activities were obtained from bacteria growing on them.

The effects of supplementing Lab-Lemco peptone–yeast extract–agar with glucose were therefore examined. The addition of glucose (0·5%) to this medium markedly increased the ribonuclease of *E. coli* B growing on it (Fig. 4*d*). An examination of young cultures in which the bacteria contained more than 0·6 μmole of RNA nucleotide/mg. dry wt.

showed that the presence of glucose did not influence the mean cell size of rapidly growing bacteria and did not significantly alter the pH of the culture. Towards maturity, however, the pH of the unsupplemented culture increased to pH 8 and that of the glucose-supplemented culture decreased to pH 5·2. The upward inclination of the curve of ribonuclease activity towards the highest concentration of RNA in the unsupplemented medium (Fig. 4*d*) could be due to low concentrations of sugars already present in the complex constituents of this medium.

If lactose or polysaccharide can exert the same influence as glucose, the wide differences in ribonuclease activity observed in *E. coli* B (Fig. 1) can largely be explained.

On the basis of the results obtained with *E. coli* B, plate-count agar was selected for general use. It supported the rapid growth of most of the species examined and it was hoped that its glucose and low salt concentration would stimulate the production of ribonuclease in those species that were able to synthesize it.

It was necessary on some occasions to lower the temperature of growth to make the time of harvesting more convenient. The lower growth rate that resulted was not expected to decrease the concentration of RNA (Wade, 1952; Schaechter, Maaløe & Kjeldgaard, 1958; Tempest & Hunter, 1965). The highest ribonuclease activities observed in *E. coli* B growing on plate-count agar at 37°, 30°, 25° and 20° were 3·3, 3·2, 2·9 and 2·7 μmoles of nucleotide released/mg. dry wt./hr. respectively, suggesting that the ribonuclease content is slightly decreased when growth temperatures lower than the optimum are used. The decrease in activity observed when the temperature is lowered from 37° to 25°, however, is barely significant.

The influence of a specific exhaustion in a major nutrient on the ribonuclease activity was examined with another ribonuclease-containing species *Aerobacter aerogenes* N.C.T.C. 418. Six preparations of this bacteria, which were kindly supplied by Dr D. Herbert and Mr D. W. Tempest, had been grown at similar rates (1 division/hr.) on a glycerol–ammonia–salts medium by continuous culture under conditions in which low concentrations of glycerol, NH_4^+, SO_4^{2-}, Mg^{2+} or K^+ were limiting the growth rate. The preparations all displayed similar high activities of ribonuclease.

A graph of the ribonuclease activity against the concentration of acetone-extracted cells of *E. coli* B (Fig. 5) suggested that a suspension of about 15% (v/v) of packed acetone-extracted bacteria was satisfactory. The maximum activity of the enzyme was not obtained at higher concentrations.

The suitability of acetone treatment as a means of obtaining killed bacteria that are freely permeable to the buffer and yet retain their ribonuclease activity was tested by comparing acetone-extracted cells of *E. coli* B and six other bacteria with preparations of disrupted cells.

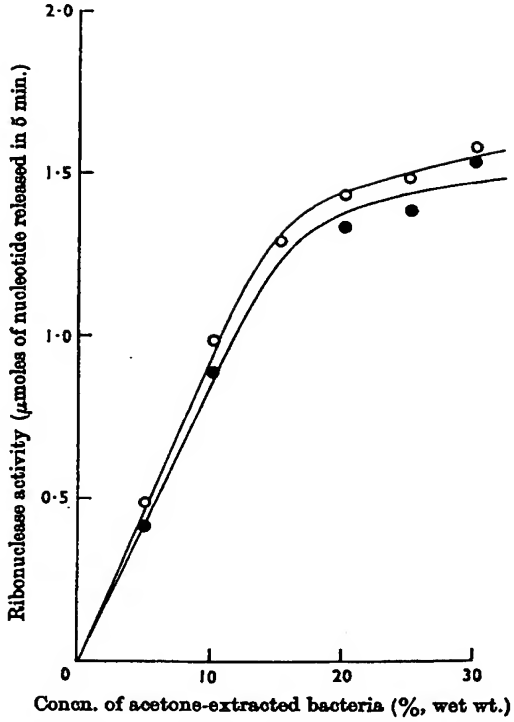

Fig. 5. Effect of the concentration of acetone-extracted bacteria on the activity of ribonuclease in *E. coli* B. The acetone-extracted bacteria were incubated at 37° in veronal–acetate–EDTA buffer, pH 7, and the ribonuclease activity was determined from the concentration of acid-soluble nucleotides released between 5 and 10 min. The results of two experiments (○, ●) are shown.

Rapidly growing bacteria were harvested from continuous cultures (Wade & Robinson, 1965*a*) and stored at −20°. One sample was extracted directly with 50% acetone, another was disrupted in a press (Hughes, 1951) and a third was disrupted and then extracted with 50% acetone. Each sample was suspended in solution containing sodium chloride (0·145M) and magnesium chloride (5mM) (about 15%, wet wt.) and incubated at 37° with veronal–acetate–EDTA buffers, pH 4–9. The concentrations of RNA in these suspensions are given in Table 3.

The results (Fig. 6 and Table 3) suggested that the three samples from each strain generally display qualitatively similar ribonuclease activities between pH 4 and pH 9 and that the sample which had been extracted directly with acetone was not deceptively low in ribonuclease activity. The samples from

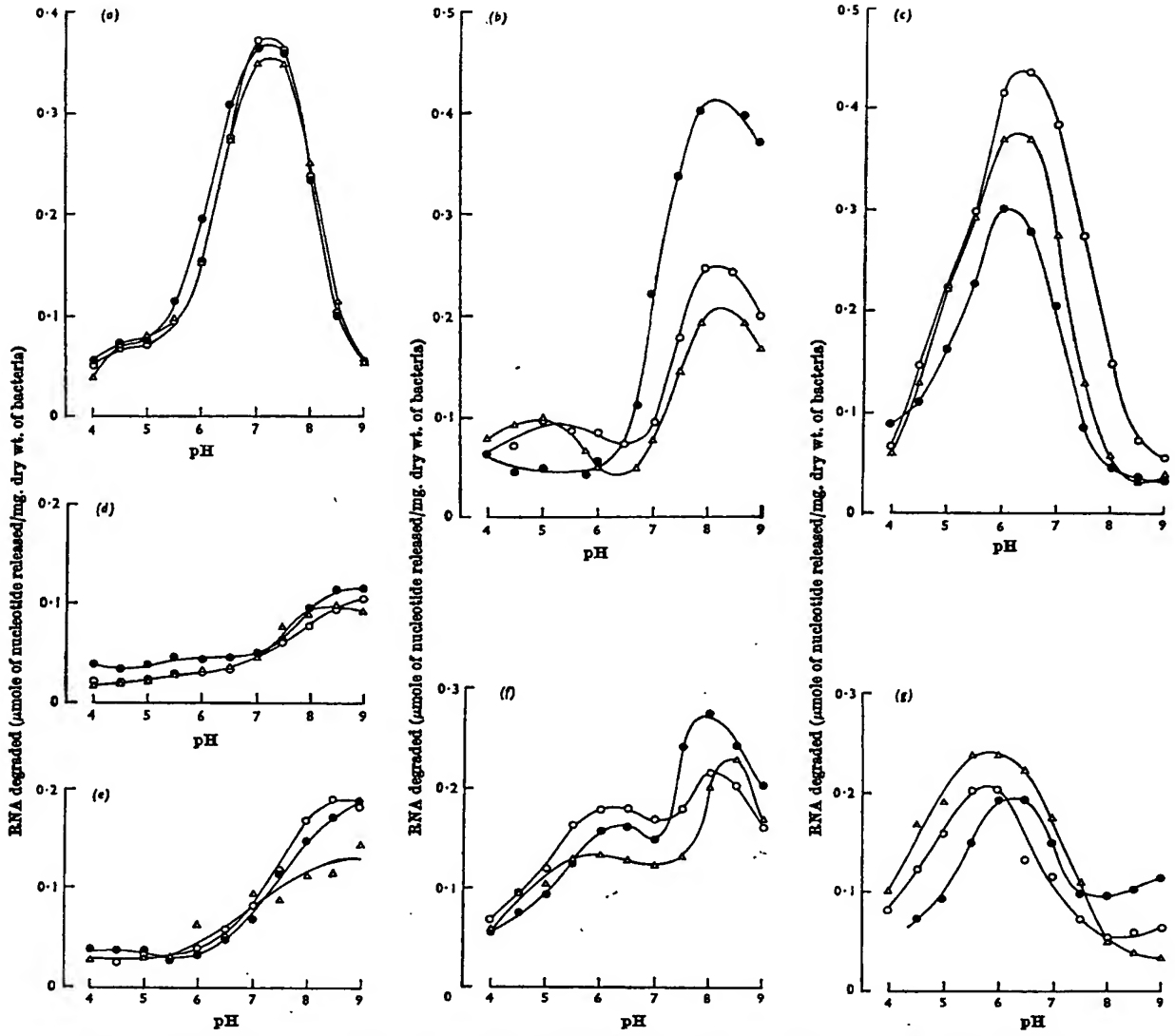

Fig. 6. Comparison between the ribonuclease activities of acetone-extracted bacteria (●), distruped bacteria (△) and disrupted acetone-extracted bacteria (○). Preparations of (*a*) *E. coli* B, (*b*) *Arthrobacter globiformis*, (*c*) *E. coli* M.R.E. 161, (*d*) *Flavobacterium aurantiacum*, (*e*) *Agrobacterium tumefaciens*, (*f*) *Corynebacterium viscosum* and (*g*) *Xanthomonas juglandis* were tested. Each preparation was suspended in solution containing NaCl (0·145 M) and $MgCl_2$ (5 mM) (15% wet wt./vol.), then diluted in veronal–acetate–EDTA buffer, pH 4–9, to about 4·5% and incubated at 37° for 10 min. (*a*), 20 min. (*d*), 30 min. (*c*), 1 hr. (*b* and *e*) or 2 hr. (*f* and *g*).

Arthrobacter globiformis showed the greatest variations (Fig. 6*b*). The maximum ribonuclease activity was significantly higher in the acetone-extracted sample than in the samples of disrupted cells. In addition, a minor ribonuclease with an optimum at about pH 5 appeared to be missing from the former. In view of its presence in both samples of disrupted bacteria (Fig. 6*b*), the most likely explanation is that this enzyme is present in the acetone-extracted bacteria but inhibited by a constituent that is retained by the cell wall.

With *E. coli* B, the concentration of harvested

Table 3. *Concentrations of RNA in the bacterial preparations examined in Fig 6*

	RNA (μmole of RNA nucleotide/mg. dry wt. of bacteria)		
	Acetone-extracted	Disrupted	Disrupted and acetone-extracted
Agrobacterium tumefaciens	0·48	0·59	0·61
Arthrobacter globiformis	0·70	0·62	0·67
Corynebacterium viscosum	0·50	0·47	0·54
Escherichia coli B	0·57	0·54	0·59
Escherichia coli M.R.E. 161	0·53	0·55	0·58
Flavobacterium aurantiacum	0·16	0·20	0·20
Xanthomonas juglandis N.C.P.P.B. 362	0·58	0·55	0·55

bacteria in the solution of 50% acetone (within the range 2–8%, wet wt.) did not affect the subsequent measurement of ribonuclease. The suspension could be stored at −20° for at least 1 month without any deterioration in enzyme activity.

The procedure finally adopted for comparing the ribonuclease activities of different species (Table 4) was based on the information gained from the study of *E. coli* B. Bacteria were normally grown on plate-count agar, extracted with 50% acetone and incubated in veronal–acetate–EDTA buffers, pH 4–9. Samples were taken before and after incubation at 37° for 5, 10, 30 and 60 min. to determine the concentration of acid-soluble nucleotides released. Graphs of these values against pH indicated the pH optimum, and a rough measure of ribonuclease activity at this pH was obtained from the maximum rate of nucleotide released. Normally in ribonuclease-containing bacteria this maximum was achieved within 10 min. of the onset of incubation. In several instances, particularly among Gram-positive species, the initial rate of breakdown was not maintained and the rate of release of nucleotides declined so that less than 50% of the RNA was ultimately degraded. In these instances the specificity of the enzyme may result in the formation of an acid-insoluble 'core' or an inhibitor may be produced during the reaction either in the form of nucleotide products or a ribosomal protein (Wade & Robinson, 1965*b*).

Plate-count agar did not support the adequate growth of some species. *Streptococcus salivarius* was grown on Lab-Lemco peptone–yeast extract–agar containing 0·1% of glucose. *Agarbacterium alginicum* and *Protaminobacter ruber* were grown on nutrient agar. *Acetobacter oxydans* was grown on a malt extract–yeast extract–glucose medium and *Azotobacter chroococcum* on a defined medium (Norris & Jensen, 1957). The two species of *Pasteurella* were grown on liquid media as described above.

The results (Table 4) showed that there are many strains of bacteria that are deficient in ribonuclease

Table 4. *Ribonucleases in different species of bacteria*

Enzyme activity is expressed as m-moles of nucleotide released/100 g. wet wt. of bacteria/hr. Laboratory strains are identified as L.S. Strains from Collections are identified by the abbreviations given in the text.

Species	Strain	pH optimum	Ribonuclease activity
Athiorhodaceae			
Rhodopseudomonas palustris	N.C.I.B. 8252	—	<1
Rhodopseudomonas spheroides	N.C.I.B. 8253	9	40
Rhodospirillum rubrum	N.C.I.B. 8740	6	1
Pseudomonadaceae			
Pseudomonas aeruginosa	L.S.	—	<1
Pseudomonas andropogoni	N.C.P.P.B. 933	7	20
Pseudomonas angulata	F.D. 496	7	2
Pseudomonas angulata	N.C.P.P.B. 79	7	4
Pseudomonas angulata	N.C.P.P.B. 214, 263 and 1237	—	<1
Pseudomonas cannabina	N.C.P.P.B. 1162	7	2
Pseudomonas chlororaphis	N.C.I.B. 9402	7	30
Pseudomonas cichorii	N.C.P.P.B. 907	—	<1
Pseudomonas delphinii	N.C.P.P.B. 650	—	<1
Pseudomonas fluorescens	KB1, N.C.I.B. 8248, 8729, 8865 and 9046	—	<1
Pseudomonas iodinum	N.C.D.O. 613	7	2
Pseudomonas lemonnieri	N.C.I.B. 8917	—	<1
Pseudomonas morsprunorum	F.D. 644	—	<1
Pseudomonas primulae	N.C.P.P.B. 133	—	<1
Pseudomonas putida	C–1B	—	<1
Pseudomonas putrefaciens	N.C.I.B. 8615	7	7
Pseudomonas putrefaciens	N.C.D.O. 756	7	40
Pseudomonas syncyanea	N.C.D.O. 759	—	<1
Pseudomonas syringae	F.D. 495	—	<1
Xanthomonas begoniae	N.C.P.P.B. 241	5–6	6
Xanthomonas campestris	N.C.P.P.B. 528	—	<1
Xanthomonas hederae	I.C.P.B. XH1	—	<1
Xanthomonas hyacinthi	N.C.P.P.B. 599	5–6	2
Xanthomonas juglandis	N.C.P.P.B. 362, I.C.P.B. XJ107	5–6	2
Xanthomonas pelargonii	I.C.P.B. XP121	6	2
Acetobacter aceti	N.C.I.B. 8554 and 8621	5	3–5
Acetobacter oxydans	N.C.T.C. 8035	5	7
Aeromonas hydrophila	N.C.M.B. 72	7	12
Aeromonas liquefaciens	N.C.M.B. 87	7	20
Azotomonas insolita	N.C.I.B. 8627	9	6
Protaminobacter alboflavus	N.C.I.B. 8167	9	8
Protaminobacter ruber	N.C.I.B. 2879	9	3
Mycoplana bullata	A.T.C.C. 4278	—	<1
Mycoplana dimorpha	N.C.I.B. 9439	9	2

Table 4 (*cont.*)

Species	Strain	pH optimum	Ribonuclease activity
Spirillaceae			
Vibrio cuneatus	N.C.I.B. 8194	7	70
Vibrio percolans	N.C.I.B. 8193	7	20
Spirillum serpens	N.C.I.B. 8658	—	<1
Azotobacteraceae			
Azotobacter chroococcum	N.C.I.B. 9125	—	<1
Rhizobiaceae			
Rhizobium leguminosarum	R.E.S. 317	9	6
Agrobacterium tumefaciens	N.C.P.P.B. 397	9	6
Chromobacterium lividum	N.C.T.C. 9796	7	3
Chromobacterium violaceum	N.C.T.C. 7150	7	3
Chromobacterium violaceum	N.C.T.C. 8685	7	100
Chromobacterium violaceum	N.C.T.C. 9370	7	40
Chromobacterium violaceum	N.C.T.C. 9373	7	140
Achromobacteraceae			
Alcaligenes faecalis	N.C.I.B. 415 and 8156	—	<1
Alcaligenes faecalis	A.T.C.C. 8748	7	2
Alcaligenes metalcaligenes	N.C.I.B. 8784	7	9
Alcaligenes metalcaligenes	N.C.I.B. 9018 and 9021	7	1
Alcaligenes viscolactis	A.T.C.C. 9086	—	<1
Alcaligenes viscosus	N.C.I.B. 8154	—	<1
Achromobacter lacticum	N.C.I.B. 8208	8	2
Achromobacter xerosis	A.T.C.C. 14780	—	<1
Flavobacterium acidificum	N.C.M.B. 683	8	10
Flavobacterium aurantiacum	N.C.I.B. 8204	8	4
Flavobacterium flavescens	N.C.I.B. 8187	8	7
Flavobacterium sauveolens	N.C.I.B. 8992	6–7	1
Agarbacterium alginicum	N.C.M.B. 886	—	<1
Enterobacteriaceae			
Escherichia aurescens	N.C.I.B. 8714	7	18
Escherichia coli	A.T.C.C. 11303, N.C.T.C. 86 and 1093, M.R.E. 162	7	24–26
Escherichia coli	w3110, N.C.I.B. 8269, N.C.T.C. 8196 and 8450, M.R.E. 164 and 165	7	14–18
Escherichia coli	N.C.T.C. 8603, 9001 and 9002, M.R.E. 163	7	8–11
Escherichia coli	C6 (N.C.I.B. 9270, N.C.T.C. 8164, M.R.E. 600)	—	<1
Escherichia coli	M.R.E. 161	5–6	8
Escherichia freundii	N.C.T.C. 8165	7–8	20
Escherichia freundii	N.C.T.C. 6071	7–8	12
Klebsiella aerogenes	N.C.T.C. 8167	7–8	10
Klebsiella aerogenes	N.C.T.C. 8172	7–8	4
Klebsiella sp.	N.C.T.C. 7242	7	1
Paracolobactrum aerogenoides	N.C.T.C. 8105	7	22
Aerobacter aerogenes	N.C.T.C. 418	7	8
Erwinia amylovora	N.C.P.P.B. 595	6	10
Erwinia carnegieana	N.C.P.P.B. 671	6	4
Serratia kellensis	N.C.T.C. 4619	6–7	3
Serratia marcescens	M.R.E. 234	6	2
Serratia marcescens	N.C.T.C. 1377	6–7	1
Proteus vulgaris	N.C.I.B. 8066 and 8067, M.R.E. 402	7–8	35–70
Proteus vulgaris	A.T.C.C. 13315, N.C.I.B. 8064, N.C.T.C. 8313	7–8	20–26
Proteus vulgaris	N.C.T.C. 401 and 4635, N.C.I.B. 8065	7–8	16–18

Table 4 (*cont.*)

Species	Strain	pH optimum	Ribonuclease activity
Enterobacteriaceae (*cont.*)			
Proteus mirabilis	PR–27	7–8	30
Salmonella arizonae	N.C.T.C. 8297	8	30
Salmonella typhimurium	M.R.E. LT/2	8	24
Shigella shigae	N.C.T.C. 4837	—	<1
Shigella flexneri	N.C.T.C. 8192	6–7	3
Brucellaceae			
Pasteurella pestis	N.C.T.C. 5924, M.R.E. 14	5–6	6–8
Pasteurella pseudotuberculosis	M.R.E. 321V and 74B	5–6	3
Brucella suis	M.R.E. 143	9	5
Micrococcaceae			
Micrococcus candidus	N.C.I.B. 8610	9	5
Micrococcus conglomeratus	N.C.I.B. 2677	8	40
Micrococcus flavus	N.C.I.B. 8166	8–9	12
Micrococcus luteus	N.C.T.C. 8512	8	4
Micrococcus lysodeikticus	Delft 538, M.R.E. 310	8–9	6
Micrococcus lysodeikticus	A.T.C.C. 4698	8–9	30
Micrococcus radiodurans	A.T.C.C. 13939	8	64
Micrococcus roseus	N.C.T.C. 7520	8–9	30
Micrococcus rubens	A.T.C.C. 186	8–9	30
Staphylococcus aureus	N.C.T.C. 6571	8–9	1
Staphylococcus epidermidis	L.S.	—	<1
Staphylococcus saprophyticus	N.C.T.C. 7291	9	2
Staphylococcus lactis	N.C.T.C. 7564 and 7944	8–9	15–22
Sarcina flava	N.C.T.C. 7743	8–9	26
Sarcina lutea	M.R.E. 304	8–9	13
Sarcina ureae	N.C.T.C. 4819	9	20
Lactobacillaceae			
Streptococcus faecalis	M.R.E. 297	9	13
Streptococcus salivarius	M.R.E. 303	9	16
Brevibacteriaceae			
Brevibacterium linens	N.C.D.O. 1002	—	<1
Kurthia zopfii	N.C.T.C. 404	9	4
Corynebacteriaceae			
Corynebacterium fasciens	N.C.P.P.B. 1488	8	40
Cornebacterium viscosum	N.C.T.C. 2416	8	20
Corynebacterium xerosis	N.C.T.C. 9755	8	40
Listeria monocytogenes	A4413 Fort Detrick	—	<1
Microbacterium lacticum	N.C.I.B. 8540	8	12
Arthrobacter aurescens	N.C.I.B. 8912	8	1
Arthrobacter citreus	N.C.I.B. 8915	8–9	32
Arthrobacter globiformis	N.C.I.B. 8907	8	8
Arthrobacter ureafaciens	N.C.I.B. 7811	8	3
Bacillaceae			
Bacillus anthracis	N.C.T.C. 8234	7–8	40
Bacillus brevis	N.C.T.C. 7577	9	6
Bacillus cereus	N.C.T.C. 945	7	14
Bacillus cereus	N.C.T.C. 2600, 6222, 6349, 7464, 9688 and 9689	7–8	36–54
Bacillus circulans	N.C.T.C. 7578	9	7
Bacillus coagulans	N.C.T.C. 3991	9	3
Bacillus lentus	N.C.T.C. 4824	9	4
Bacillus licheniformis	L.S.	9	9
Bacillus megaterium	N.C.T.C. 2607 and 6347	9	5
Bacillus polymyxa	L.S.	9	8
Bacillus pumilus	N.C.T.C. 7576, A.T.C.C. 6631	9	5–8
Bacillus sphaericus	N.C.T.C. 6348 and 9602	9	5–7
Bacillus sphaericus	N.C.T.C. 7582	9	14
Bacillus subtilis	L.S., A.T.C.C. 9372	9	12–18
Mycobacteriaceae			
Mycobacterium smegmatis	N.C.T.C. 8159	9	5
Mycobacterium phlei	N.C.T.C. 8151	9	1

Table 4 (*cont.*)

Species	Strain	pH optimum	Ribonuclease activity
Actinomycetaceae			
Nocardia erythropolis	N.C.I.B. 9158	9	16
Streptomycetaceae			
Streptomyces thermoviolaceus	R.E.S. A71	8	1
Streptomyces griseoflavus	R.E.S. A77	8	1
Micromonospora vulgaris	R.E.S. A64	—	<1
Thermopolyspora glauca	R.E.S. A66	8–9	1

and, when such an enzyme is present, its pH optimum affords a means of distinguishing it from other ribonucleases.

DISCUSSION

Bacterial ribonucleases have received little attention in the past and, where they have been described, the wide variety of conditions used for their assay, particularly with respect to the concentration of Mg^{2+}, makes a detailed comparison with the present results of little value.

The examination of bacterial ribonucleases reported here has shown that a wide variety of species are deficient in this type of enzyme and that the pH optimum of enzymes in the remainder are frequently characteristic of the genus. The number of species examined is too small to evaluate fully the use of this character in taxonomy. In a few instances, however, the number of species and strains of a genus that have been examined are sufficiently large to justify some comment.

Most of the pseudomonads have strains that contain negligible ribonuclease; the remainder have neutral enzymes (Table 4). Three of these, *Ps. andropogoni*, *Ps. chlororaphis* and *Ps. putrefaciens* N.C.D.O. 756, have very active enzymes and closely resemble the *Vibrio* genus in this respect. The distinction between these two genera is not well defined (Shewan, Hodgkiss & Liston, 1954).

In view of the close similarity between pseudomonads and xanthomonads in other features, it is surprising that the pH optima of the ribonucleases should be different. The close physiological resemblance between *Aeromonas* and *Paracolobactrum*, on the other hand (Breed *et al.* 1957), extends to the ribonucleases; both have moderately active neutral ribonucleases (Table 4). The absence of ribonuclease from *Alcaligenes faecalis* has also been observed by Dr M. Teuber (personal communication).

E. coli usually has a neutral ribonuclease (Table 4 and Fig. 6*a*). The absence of this enzyme from strain C6 (N.C.T.C. 8164, N.C.I.B. 9270 and M.R.E. 600), which is the neotype for *E. coli* type II in the classification of water-borne bacteria, is not in any way connected with the reactions on which this classification is based. Seventeen other strains of the same type, kindly supplied by Dr N. P. Burman (Metropolitan Water Board, London), had normal amounts of ribonuclease. *E. coli* M.R.E. 161 was isolated from sewage effluent. Since its identification was based on elementary medical bacteriology it could be a species of *Erwinia*, which would account for the low pH optimum of its ribonuclease (Fig. 6*c* and Table 4).

The ribonucleases of all the strains of *Proteus vulgaris* examined were similar in pH optimum and no correlation between high ribonuclease activity and motility was observed (Wade & Robinson, 1965*b*).

Unlike most of the Gram-negative bacteria, most of the Gram-positive bacteria have ribonucleases with alkaline pH optima. The staphylococci generally have a weak ribonuclease or none at all (Table 4). *Staphylococcus lactis*, which appears to be the exception, is regarded by some bacteriologists as a *Micrococcus*. The absence of a ribonuclease from *Staph. epidermidis* has also been observed by Dr L. A. Heppel (personal communication).

As in several other features, *Bacillus cereus* and *Bacillus anthracis* resemble each other in the possession of a very active neutral ribonuclease, which distinguishes them from the other species that have been examined (Table 4).

The results of the more detailed examination of ribonucleases from *Arthrobacter globiformis* (Fig. 6*b*) and *Corynebacterium viscosum* (Fig. 6*f*) serve as a warning that more than one ribonuclease may be present in the same species. Two distinct ribonucleases with similar pH optima have been demonstrated in *E. coli* (Neu & Heppel, 1965; Anraku & Mizuno, 1965). The single pH optimum most frequently observed and recorded may in fact be the unresolved activities of more than one enzyme.

The absence of a detectable ribonuclease from a bacterial cell is clearly not confined to *Ps. fluorescens* N.C.I.B. 8248 (Wade & Robinson, 1963) and is not in fact an unusual occurrence (Table 4). The function of Mg^{2+}-independent ribonuclease is obviously not a vital one, although it may confer some benefit that is not immediately apparent.

Where the enzyme is present, the distinction between ribonucleases in this paper has been confined to the pH optima, though another feature that affords a measure of distinction is the specificity of the enzyme.

Some bacterial ribonucleases are secreted during growth. In those species where this occurs the recorded activities (Table 4) will not fully reflect the capacity of the bacteria to produce the enzyme. To what extent the ribonucleases of the species listed in Table 4 are extracellular is not yet known.

We are grateful to Mr W. J. Brent for technical assistance and to Dr D. Herbert for his advice and interest.

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J Periodont Res 2006; 41: 554–559

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00905.x

Cytokine production in human periodontal ligament cells stimulated with *Porphyromonas gingivalis*

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Yamamoto T, Kita M, Oseko F, Nakamura T, Imanishi J, Kanamura N. Cytokine production in human periodontal ligament cells stimulated with Porphyromonas gingivalis. *J Periodont Res 2006; 41: 554–559.* © Blackwell Munksgaard 2006

Background and Objective: Although some functions and characterizations of human periodontal ligament (hPDL) cells have been reported, the role of hPDL cells in periodontal disease is poorly understood. We have previously reported that hPDL cells produce many kinds of inflammatory cytokines by stimulation with *Prevotella intermedia*. In this study, we examined the production of cytokines in hPDL cells stimulated with *Porphyromonas gingivalis* as compared with *P. intermedia*.

Material and Methods: hPDL cells cultured in Dulbecco's modified Eagles's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. After three to four passages, hPDL cells were stimulated with *P. intermedia* (ATCC25601) or *P. gingivalis* (ATCC33277) for 24 h. Total RNA was extracted by ISOGEN and the expression of cytokine mRNA was determined using reverse transcription–polymerase chain reaction. Cytokines in the culture supernatants were assessed by enzyme-linked immunosorbent assay.

Results: The expression of interleukin-1β, interleukin-6, interleukin-8, tumor necrosis factor-α (TNF-α), receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) mRNA was detected in hPDL cells after stimulation with *P. gingivalis* as well as *P. intermedia*. There were no significant differences in the kind of cytokines expressed in hPDL cells between *P. gingivalis* and *P. intermedia*. However, *P. gingivalis* induced a significantly higher production of cytokines in hPDL cells than *P. intermedia* ($p < 0.05$).

Conclusion: This study demonstrated that hPDL cells produce many kinds of cytokines as a result of bacterial stimulation, including stimulation with *P. gingivalis* and *P. intermedia*. These results suggest that hPDL cells may play a role in cytokine production in periodontal disease.

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Key words: immunology; pathogenesis; periodontal ligament cells; periodontium

Accepted for publication March 23, 2006

Various factors are known to be involved in the mechanism of periodontal tissue destruction in periodontitis. In particular, the presence of periodontopathogenic bacteria is a direct cause of periodontal tissue destruction. Chronic bacterial stimulation of periodontal tissue causes persistent or excessive periodontal tissue breakdown, with hard tissue destruction. Thus, periodontitis occurs in the presence of persistent dental plaque infection in the special environment of the periodontal pocket. Inflammatory cells and osteoclasts are considered to be chronically activated, leading to the loss of attachment and alveolar bone destruction (1).

In recent years, several immunological studies have reported on periodontal tissue breakdown, using inflammatory exudates in the gingival crevice (2,3), gingiva (4) and periodontal ligaments (5,6). However, much of the function and structure of human periodontal ligament (hPDL) cells, consisting of fibroblasts, osteoblasts and osteoclasts, is unknown (7). Typical bacteria that form dental plaques in the periodontal pocket include *Porphyromonas gingivalis* and *Prevotella intermedia* (8,9). It has been reported that *P. gingivalis* leads to periodontal tissue destruction and alveolar bone resorption through interleukin-6 and interleukin-8 release from hPDL cells (10), and these inflammatory cytokines play a role in the destruction and disintegration of the extracelluar matrix (11). We have previously reported that hPDL cells may play an important role as cells producing inflammatory cytokines in periodontal diseases (12). In addition, hPDL cells stimulated with *Escherichia coli* lipopolysaccharide induce both receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) expression, which relate with bone metabolism, by up-regulating interleukin-1β and tumor necrosis factor-α (TNF-α) (5). Furthermore, it has been reported that *P. gingivalis* inhibits fibroblast growth more strongly than *P. intermedia* (13), suggesting that the bacteria differ in their pathogenicity. However, the effect of periodontopathogenic bacteria on the expression of cytokines in hPDL cells has not been reported.

In this study, we examined the ability of hPDL cells to produce cytokines, mainly those involved in inflammation and bone metabolism, after stimulation by the periodontopathogenic bacteria, *P. gingivalis* and *P. intermedia*.

Materials and methods

Preparation of hPDL cells

hPDL cells were obtained from a healthy erupted maxillary third molar from three donors (a 21-yr-old woman, a 23-yr-old man and a 24-yr-old woman), removed for orthodontic reasons and used with informed consent. The tissue was minced and cultured as explants in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) containing 10% fetal bovine serum (FBS) and antibiotics, as previously described (12,14). After three to four passages, the cells were used for experiments. This experimental procedure was approved by the Ethics Committee, Kyoto Prefectural University of Medicine.

Preparation of bacteria

P. intermedia (ATCC25611) and *P. gingivalis* (ATCC33277) are gram-negative spiral rods and *Streptococcus mutans* (*FA1*) is a gram-positive coccus. All bacteria were generously supplied by the Department of Microbiology, Kyoto Prefectural University of Medicine Graduate School of Medical Science. *P. intermedia* and *P. gingivalis* were grown anaerobically on 5% sheep blood agar plates (Nissui Pharmaceutical, Tokyo, Japan) at 37°C for 24 h.

Stimulation with bacteria in hPDL cells

The hPDL cells were seeded onto Petri dishes at a concentration of 1×10^6 cells/dish. The cells reached confluence after approximately 1 wk of culture, and then were stimulated with *P. intermedia*, *P. gingivalis* or *S. mutans* [1×10^7 colony-forming units (CFU)/ml] for 24 h.

Expression of cytokine mRNA

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), and the expression of cytokine mRNA was determined by using reverse transcription–polymerase chain reaction (RT-PCR). We analyzed the mRNA expression of interleukin-1β, interleukin-6, interleukin-8, TNF-α, receptor activator of nuclear factor-κB (RANK), RANKL and OPG. β-actin was used as an internal control. The primer sequences are shown in Table 1. For RT–PCR, we used our previously reported procedure (15). Briefly, total RNA was extracted with ISOGEN, and cDNA was produced using Superscript RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), oligo dT primer and 2.5 mmol/l dNTP Mixture (Takara Shuzo, Otsu, Japan). PCR was performed for 35 cycles, each consisting of 1 min at 95°C for denaturation, 1 min at 50°C for annealing and 1 min at 72°C for extension. Ten microliters of each PCR product was analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide (Bio-Rad Laboratories, Hercules, CA, USA), and the bands were visualized under ultraviolet (UV) light.

Production of cytokine in hPDL cells

Assays for cytokines in the culture supernatants employed commercially available enzyme-linked immnosorbent assay (ELISA) kits obtained from the following sources: interleukin-1β, interleukin-6, interleukin-8, TNF-α (Biosource International, Camarillo, CA, USA), RANKL and OPG (Cosmo Bio, Tokyo, Japan). All assays were conducted in accordance with the manufacturer's instructions.

Morphologial changes of the cells

Before and after the stimulation with bacteria, the morphological changes of hPDL cells were observed using an inverted optical microscope (Olympus, Tokyo, Japan). The size of the cells was assessed viaually. The viability of hPDL cells was examined using the Trypan Blue exclusion test.

Statistical analysis

Data obtained from separate experiments were pooled and expressed as means ± standard error of the mean (SEM). Comparisons between groups were made using the Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Expression of cytokine mRNA in hPDL cells

To examine the ability of *P. gingivalis* to induce the production of cytokines,

Table 1. Polymerase chain reaction primer, sequences and predicted size

Cytokine		Sequences	Predicted size
Interleukin-1β	Sense	ATAAGCCCACTCTACAGCT	443
	Antisense	ATTGGCCCTGAAAGGAGAGA	
Interleukin-6	Sense	GTACCCCCAGGAGAAGATTC	819
	Antisense	CAAACTGCATAGCCACTTTC	
Interleukin-8	Sense	GCTTTCTGATGGAAGAGAGC	585
	Antisense	GGCACAGTGGAACAAGGACT	
TNF-α	Sense	TCGGGCCAATGCCCTCCTGGCCAA	468
	Antisense	GTAGACCTGCCCAGACTCGGCAAA	
RANK	Sense	TTAAGCCAGTGCTTCACGGG	497
	Antisense	ACGTAGACCACGATGATGTCGC	
RANKL	Sense	CAGCACATCAGAGCAGAGAAAGC	517
	Antisense	CCCCAAAGTATGTTGCATCCTG	
OPG	Sense	GTACGTCAAGCAGGAGTGCAATC	472
	Antisense	TTCTTGTGAGCTGTGTTGCCG	
β-actin	Sense	GTGGGGCGCCCCAGGCACCA	541
	Antisense	CTCCTTAATGTCACGCACGATTTC	

OPG, osteoprotegerin; RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; TNF-α, tumor necrosis factor-α.

cytokine-specific expression of mRNA in hPDL cells was analyzed using RT–PCR.

No expression of interleukin-1β, interleukin-6, interleukin-8, RANK, RANKL or OPG mRNA was observed in the control cells before stimulation with bacteria, whereas in stimulated hPDL cells, interleukin-1β, interleukin-6, interleukin-8, TNF-α, RANKL and OPG mRNA were detected (Fig. 1). However, there were no significant differences in the types of cytokines expressed in hPDL cells upon stimulation with either *P. intermedia* or *P. gingivalis*. Each experiment was performed eight or nine times. We carried out the same experiment using other hPDL cells, and obtained the same results.

Production of cytokine in hPDL cells

To examine the production of cytokines after stimulation with *P. intermedia* or *P. gingivalis*, hPDL cells were incubated with either *P. intermedia* or *P. gingivalis* for 24 h, and the culture supernatants were analyzed by ELISA. The production of interleukin-1β, interleukin-6, interleukin-8 and RANKL increased significantly after stimulation with *P. intermedia*. On the other hand, *P. gingivalis* induced significantly higher production of interleukin-1β, interleukin-8, TNF-α and RANKL in PDL cells than *P. intermedia* and *S. mutans* ($p < 0.05$, Fig. 2), but the production of OPG was significantly lower ($p < 0.05$) (data not shown).

Morphological changes of the hPDL cells

Bacterial stimulation caused no changes in the morphology of hPDL cells, and did not affect cellular viability (data not shown). hPDL cells did not exhibit morphological changes (Fig. 3).

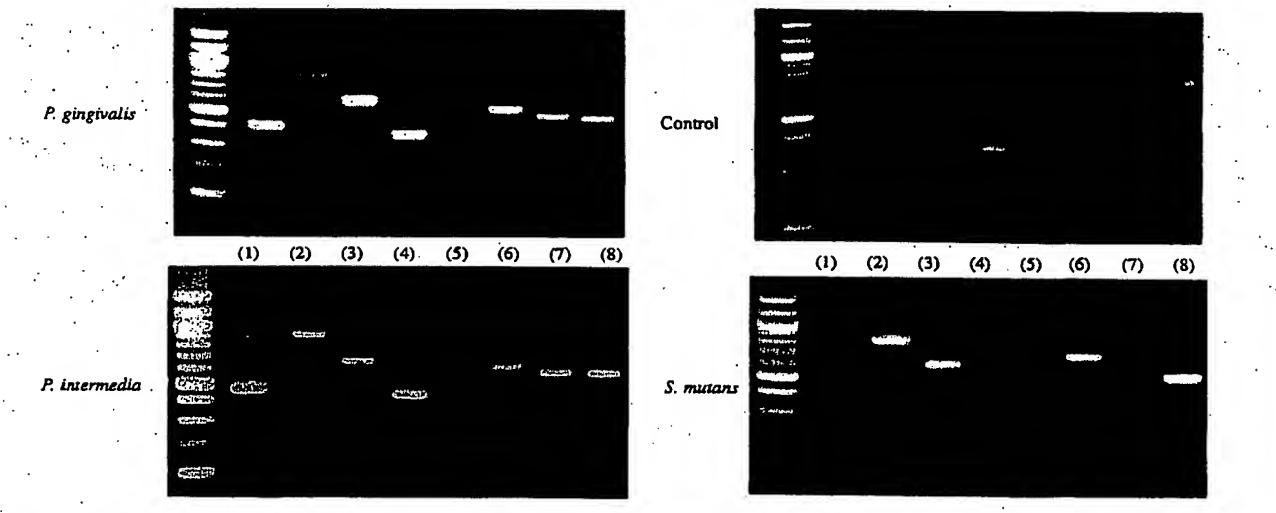

Fig. 1. Expression of cytokine mRNA in in human periodontal ligament (hPDL) cells stimulated with *Porphyromonas gingivalis*, *Prevotella intermedia* or *Streptococcus mutans*. The expression of interleukin-1β, interleukin-6, interleukin-8, receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) in hPDL cells stimulated with *P. gingivalis*, *P. intermedia* or *S. mutans* is shown. Lane 1, interleukin-1β; lane 2, interleukin-6; lane 3, interleukin-8; lane 4, tumor necrosis factor-α (TNF-α); lane 5, receptor activator of nuclear factor κB (RANK); lane 6, receptor activator of nuclear factor κB ligand (RANKL); lane 7, osteoprotegerin (OPG); and lane 8, β-actin. Unmarked lane, molecular weight markers.

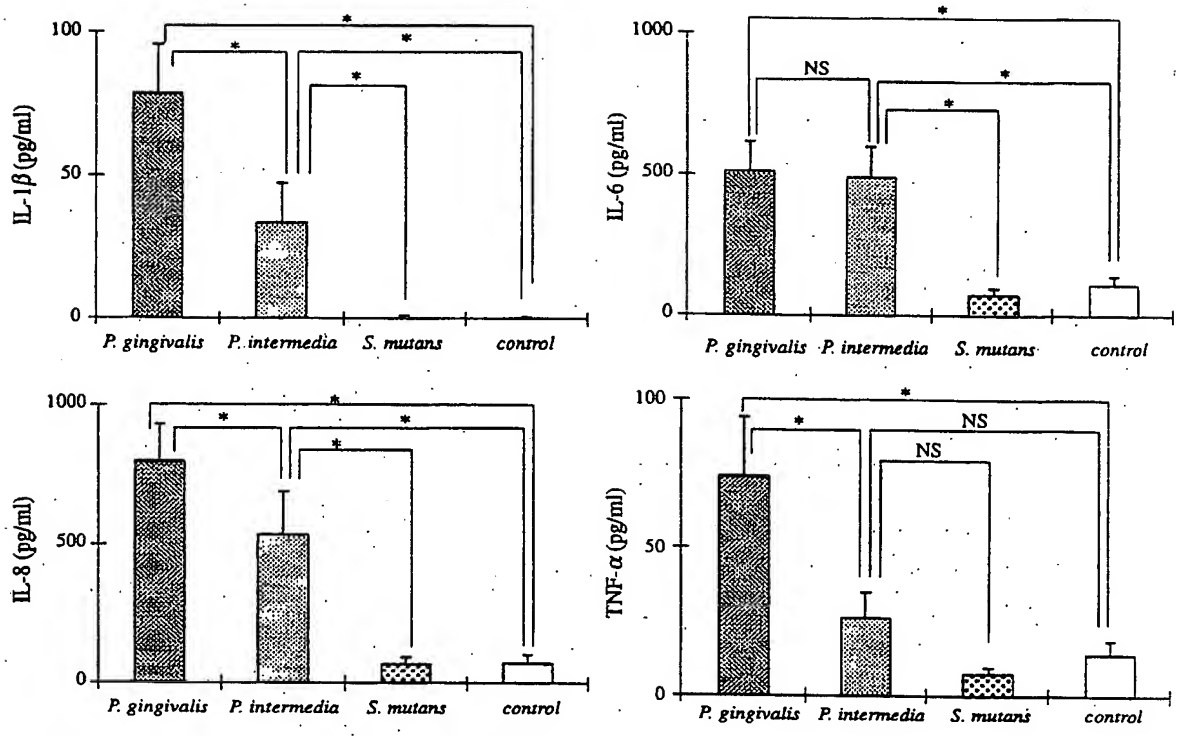

Fig. 2. Effect of *Porphyromonas gingivalis*, *Prevotella intermedia* or *Streptococcus mutans* on the production of inflammatory cytokines. The amount of inflammatory cytokines on human periodontal ligament (hPDL) cells stimulated with *P. gingivalis* was significantly higher than the amount of inflammatory cytokines stimulated by *P. intermedia*. Results are expressed as mean ± standard error (SE). $^*p < 0.05, n = 11$. IL, interleukin; TNF-α, tumor necrosis factor-α.

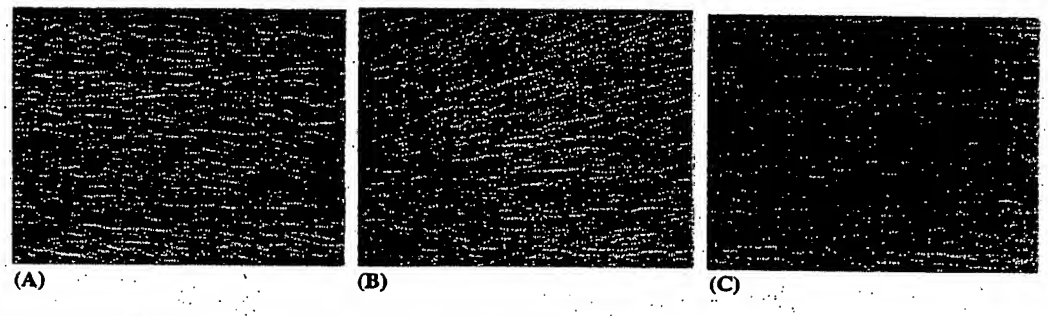

Fig. 3. Photomicrographs of human periodontal ligament (hPDL) cells stimulated with *Porphyromonas gingivalis* (A), *Prevotella intermedia* (B) and control (C). hPDL cells showed no morphological changes following stimulation with *P. gingivalis* or *P. intermedia*. (Original magnification ×100).

Discussion

The gram-negative, black-pigmented bacteria *P. gingivalis* and *P. intermedia* used in this study are typical adult periodontal disease-causing bacteria that form dental plaques in the periodontal pocket. Van Winkelhoff *et al.* reported that *P. gingivalis* and *P. intermedia* were significantly more prevalent in patients with periodontal destruction, and *P. gingivalis* is the strongest bacterial marker for destructive periodontal disease (8). Tanaka *et al.* showed that the number of both bacteria was greater in the area of chronic inflammation than in the area of acute exacerbation of chronic inflammation in adult periodontal disease (16). However, it is well known that the presence alone of periodontopathogenic bacteria, such as *P. gingivalis*, *P. intermedia* and *Actinobacillus actinomycetemcomitans*, is insufficient for the development of periodontal disease and is only a risk factor for periodontal disease (17).

Therefore, it is necessary to examine the effect of both species of bacteria on hPDL cells to elucidate the process of periodontal tissue breakdown. On the other hand, it is well known that dental caries is caused by *S. mutans*. Van der Reijden *et al.* reported a negative correlation between the percentage of *S. mutans* and the percentage of *P. gingivalis* in subgingival plaque (18). Thus, *S. mutans* is thought to be non-periodontopathogenic against PDL cells in the periodontal pocket. In the present study, we examined the ability of *P. gingivalis* to induce the production of cytokines in hPDL cells. In hPDL cells, TNF-α mRNA was constitutively detected before and after the stimulation of these bacteria. However, the expression of interleukin-1β, interleukin-6, interleukin-8, RANKL and OPG was induced in response to exposure to these bacteria in hPDL cells. These results indicate that hPDL cells are involved in periodontal tissue inflammation.

It has been reported that hPDL cells stimulated with *P. gingivalis* lipopolysaccharide induced the expression of inflammatory cytokine mRNA (10), and that the expression of inflammatory cytokine mRNA was enhanced by costimulation with *P. gingivalis* lipopolysaccharide and excessive mechanical stress (19). Our results are in agreement with their results, and we demonstrated that *P. gingivalis* and *P. intermedia* induce cytokine production not only at the mRNA level, but also at the protein level.

RANKL and OPG in hPDL cells are known to play important roles in the differentiation of osteoclats (20). It has been reported that more severe periodontal disease was associated with a greater amount of RANKL and a smaller amount of OPG from gingival crevicular exudates in periodontal disease patients (21). In the present study, *P. gingivalis* induced a higher production of RANKL than *P. intermedia*, suggesting that *P. gingivalis* has a higher pathogenicity for hPDL cells than *P. intermedia*. Using lipopolysaccharide from different strains of *P. gingivalis*, Shipira *et al.* reported that different strains of *P. gingivalis* had different levels of pathogenicity (22). Thus, more careful study is needed to elucidate the relationship between the inducibility of RANKL or OPG and the pathogenicity of *P. gingivalis*.

Furthermore, Kon *et al.* reported that in a mouse model of iliac bone fracture healing, interleukin-1β and TNF-αmRNA, in addition to RANKL, OPG and macrophage colony-stimulating factor (M-CSF) mRNA, were expressed in the early and late stages of healing (23). Nukaga *et al.* found that interleukin-1β stimulation of hPDL cells increased RANKL mRNA expression (24). To date, we have shown that TNF-α mRNA is involved in the expression of RANKL and OPG mRNA in the wall of peri-apical cysts (13). These observations suggest that inflammatory cytokines are closely related to the expression of RANKL and OPG mRNA.

hPDL cells did not show morphological changes when cocultured with *P. gingivalis* or *P. intermedia*, which is in agreement with the report that oral infections, such as periodontal disease and caries, are caused by low-virulence bacteria (25). As shown in Fig. 1, the hPDL cells used in this study appeared morphologically to be fibroblasts (7); however, we consider that hPDL cells should be separated into fibroblasts, osteoblasts and osteoclasts in future studies, because osteoblasts and osteoclasts are also expected to be involved in the pathogenesis of periodontal disease.

In conclusion, we demonstrated that hPDL cells could produce many types of cytokines by stimulation with *P. gingivalis and P. intermedia*. These results suggest that hPDL cells may play a role in cytokine production in periodontal disease.

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